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Our studies, together with work carried out in other laboratories, have indicated that defects in the Rb-Cyclin D1-p16 cell cycle regulatory system are present in the vast majority of breast cancer cells. In previous years we have focused on characterizing these defects and on determining the mechanisms responsible for causing them. In the current year we have turned our efforts towards demonstrating the contribution of these defects to the tumorigenic properties of breast cancer cells, in order to provide potential targets for therapy. In particular, we have focused on the effects of restoring the expression of the tumor suppressor p16 in breast cancer cells, using constructs in which p16 expression in under control of the inducible Tet promoter. After demonstrating the feasibility of this approach in breast cancer cells in transiently transfected cells, we have now produced stably transfected cell lines in order to test effects of p16 expression on growth in soft agar and in formation of tumors in nude mice. Currently we have demonstrated that MCF-7 breast cancer cells stably transformed with constructs expressing p16 are impaired or have lost the ability to grow in soft agar, indicating that defects in p16 expression may make a critical contribution to tumorigenicity. However, additional controls are required to establish unequivocally that loss of growth of these cells in soft agar is in fact due to restoration of p16 expression.

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T. R. Longo 10/19/98

PI - Signature Date

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Introduction

The present report covers progress made in the fourth year of the grant. We have requested and received a six month extension of the grant period (until April 22, 1999, research to be completed by March 22, 1999) and will submit our final report at that time.

During the first four years of the grant, as described in previously and in the present annual report, we have carried out studies which, along with work in other laboratories, have established that one or more defects in the Rb (retinoblastoma) protein – cyclin D1 – p16 (multiple tumor suppressor 1) cell cycle regulatory system are present in a large majority of breast cancer cells. We have investigated the mechanisms that bring about defects in the expression and function of these regulatory components in breast cancer, and have now in the current year turned our major effort towards demonstrating the contribution of these defects to the tumorigenicity of breast cancer cells, in order to provide potential targets for therapeutic control of this tumorigenicity.

Knowledge of the mechanisms of cell cycle regulation and the techniques available to investigate it have undergone very large advances since our original proposal was written. We have accordingly altered and expanded our original goals, in order to bring our investigation into currency with the present state of knowledge, as reflected in the revised Statement of Work submitted in the third year of the grant. In particular, technology for the transfection and expression of genes in cells has advanced considerably, and we have recently chosen to exploit one of those advances for the introduction and control of expression of genes affecting cell cycle regulation in breast cancer cells. In addition, the investigation of expression of the tumor suppressor p16 and its interaction with the cyclin D1 – Rb regulatory axis, which was unknown at the time the grant was written, has been incorporated as a major aspect of our investigation, since to omit the contribution of p16 would render our studies seriously incomplete.

Materials and Methods

Breast cancer cell lines and tumor material

Two breast cancer cell lines, MCF-7, and ZR75.1, and one normal, SV40-transformed breast epithelial cell line, HBL-100 were obtained from the University of Colorado Tissue Culture Core Facility. The cell lines were cultured in MEM media supplemented with 10% fetal calf serum, 0.2% sodium bicarbonate, 10 mM HEPES, 1% non-essential amino acids, 2 mM L-glutamine and 6 ng/ml insulin.

Antibodies

The following primary antibodies were obtained from Upstate Biotechnology, Lake Placid, NY: anti-cyclin A, anti-cyclin B1, and anti-cyclin D1. Anti-cyclin E and anti-p16 were obtained from Pharmingen. Anti-pRb was a gift from Dr. Wen-Hua Lee (University of Texas Health Science Center, San Antonio, TX). The horseradish peroxidase-conjugated secondary antibodies were obtained from Bio-Rad.

Oligonucleotides

The pUDH15-1 oligonucleotide primer sequences are as follows:

Sense primer: 5' TAG ATG TGC TTT ACT AAG TC 3'
Antisense primer: 5' ACT TGA TGC TCT TGA TCT TC 3'

The p16 exon 2 oligonucleotide primer sequences are from Hussussian et al. (1994).

Protein extraction and western blot analysis

Cells were harvested, washed in PBS, and resuspended in Laemmli sample buffer (Laemmli., 1970). The extracts were then boiled for 4 minutes, sheared through a 26-gauge syringe needle, aliquoted, and stored at -80°C.

Approximately 100 µg of each protein extract were subjected to SDS/PAGE and transferred either to nitrocellulose (Schleicher and Schuell) or Immobilon P (Amersham) membranes for 45 minutes at 0.45 A using the Genie Electrophoretic Blotter (Idea Scientific, Minneapolis). Membranes were stained with Ponceau dye to control for equal loading and immunodetection performed using the enhanced chemiluminescence (ECL) kit (Amersham) according to the manufacturer's instructions.

Fluorescent activated cell sorter (FACS) analysis

Cells were harvested and washed twice in PBS. A minimum of 3×10^3 cells were then resuspended in 0.5 ml Krishan's stain (Krishan, 1975) and subjected to cell cycle distribution analysis by the FACS core of the Cancer Center, University of Colorado Health Sciences Center.

DNA isolation

DNA was prepared by incubating cells or finely minced solid tissues at 55°C in lysis buffer (10 mM Tris pH 8.0, 2.0 mM EDTA pH 8.0, 10 mM NaCl, 5% SDS) containing 1

mg/ml Proteinase K. The samples were then subjected to two phenol-chloroform extraction's and one chloroform:isoamylalcohol (24:1) extraction, followed by ethanol precipitation.

DNA analysis by Methylation-specific PCR (MSP)

DNA samples were modified with sodium bisulfite according to the method of Herman et al. (1996), and precipitated with ammonium acetate (3M final concentration) and two volumes of ethanol. The resulting templates were subjected to PCR using oligonucleotides designed from the promoter of the p16 gene (Herman et al., 1996) specific for wildtype, methylated or unmethylated DNA. A 20 µl reaction mixture overlaid with a drop of mineral oil contained a final concentration of 20 ng of genomic DNA, 120 ng of each oligonucleotide, PCR buffer supplied by Promega (10 mM tris-HCL, pH 8.3; 5 mM KCl; 0.1% gelatin); 200 µM dNTPs; 1.5 mM $MgCl_2$; and 0.06 units μl^{-1} Taq polymerase (added once the reaction temperature reached 95°C). The DNA was subjected to 35 cycles of amplification consisting of denaturation for 0.5 minutes at 94°C, annealing for 0.5 minutes at 60°C (for unmethylated-specific oligonucleotides) or 65°C (for wildtype- and methylated-specific oligonucleotides), and elongation for 0.5 minutes at 72°C, followed by a final elongation step of 10 minutes at 72°C. The PCR products were subjected to electrophoresis on a 2% agarose gel, stained with ethidium bromide and visualized with uv light.

Identification of pUDH15-1 and p16 stable transfectants by PCR analysis

An approximately 50% confluent $25cm^2$ flask of each clone was vigorously shaken to detach cells undergoing mitosis. These cells were collected by centrifugation and lysed in a solution of 10 mM Tris HCL pH 8.7, 50 mM KCl, 1.2 mM $MgCl_2$, 0.01% gelatin, 0.45% NP-40, 0.45% Tween-20 and 10 mg/ml proteinase K at 55°C for 60 minutes. Five microliters of each of the resulting cellular lysates were subjected to PCR analysis using oligonucleotide primers corresponding to the appropriate plasmid in the following manner: A 20 µl reaction mixture overlaid with a drop of mineral oil contained 5 µl genomic DNA, 0.2 µM of each primer, PCR buffer supplied by Promega (10 mM tris-HCL, pH 8.3; 5 mM KCl; 0.1% gelatin); 200 µM dNTP's; 1.5 mM $MgCl_2$; and 0.06 units μl^{-1} Taq polymerase. Following an initial denaturation period of 7 minutes at 95°C, the DNA was subjected to 30 cycles of amplification consisting of denaturation for 1 minute at 94°C, annealing for 1 minute at 52°C (for pUDH15-1reactions) and 58°C (for p16 reactions), elongation for 1 minute at 72°C, and a final elongation step of 10 minutes at 72°C. The PCR products were subjected to electrophoresis on a 2 % agarose gel, stained with ethidium bromide and visualized with ultraviolet (uv) light.

Transfection of breast cancer cell lines with Tet plasmids

For both transient and stable transfections, cells were grown in 35 mm plates until they reached 50-60% confluence. At this time, 2.4 µg of each plasmid(s) was mixed with a total of 12 µl of lipid per well in serum-free medium, and incubated at room temperature for 15 minutes. The cells were then washed with 1 ml of serum-free medium and overlaid with the DNA/lipid mixture. After 5 hours at 37°C, the DNA/lipid mixture was removed and replaced with medium supplemented with 10% serum, 500 µg/ml G418 plus or minus 1 µg/ml doxycycline. For transient transfection, the cells were grown for a further 24 hours at 37°C

and harvested for p16 protein analysis. For stable transfections, the cells were grown for a further 48 hours then selected for growth in medium supplemented with 500 μg/ml G418 and 500 μg/ml zeocin plus or minus 1 μg/ml doxycycline. Individual zeocin-resistant colonies were isolated using sterile cloning rings (Bellco) and cultured in selection media for further analysis.

Soft agar analysis

Five milliliters of a mixture containing 0.4% agar, 10% serum, 1 X MEM medium, 500 μg/ml G418, 500 μg/ml zeocin, plus or minus 1 μg/ml doxycycline (previously incubated at 45°C) were added to each of 64 60 mm plates, and allowed to harden at room temperature. Each of the transfectants, MCF-7/15-1/p16#29, MCF-7/15-1/p16#69, and MCF-7/15-1/p16#13, that had been grown in the presence or absence of 1 μg/ml doxycycline in two 75 cm^2 flasks for 7 days, were harvested and counted. The cells were resuspended in MEM medium supplemented with 10% serum to 1.25 X 10^4 cells/ml, and 4 ml of each suspension added to each of two tubes containing 6 ml of the above agar/medium mixture. After mixing, 2.5 ml of the cell-agar suspension were plated on to 8 of the 60 mm plates containing a base layer of agar/medium. The MCF-7 parent cell line was also grown in the presence and absence of doxycycline for 7 days and subject to soft agar analysis in the absence of G418 and zeocin selection.

Results

Regulated expression of p16 in breast cancer cell lines lacking endogenous p16 protein

As discussed in our previous progress reports, the most frequent cell cycle aberration observed in breast cancer cell lines and tumor tissues involves the combined inactivation of p16 and overexpression of cyclin D1. The loss of p16 expression results in the constitutive activation of cyclin D1/CDK4/CDK6 complexes that phosphorylate and thereby inactivate pRb, thus allowing cells unregulated entry into S-phase.

To determine the effect of p16 expression upon the transformed and tumorigenic properties of breast cancer cell lines that lack endogenous p16 protein and show 10-fold overexpression of cyclin D1 (MCF-7 and ZR75.1), we have employed the Tet-Off System (Gossen et al., 1995). The construction of stable "Tet" clones expressing p16 in a regulated manner will be described later. However prior to the isolation of "Tet" clones we tested the MCF-7 and ZR75.1 cells for sensitivity to p16 function by infecting them with an adenoviral construct, Ad-p16 (Introgen Therapeutics) that constitutively expressed the p16 gene under the control of a CMV promoter (Schrump et al., 1996).

Infection of MCF-7 and ZR75.1 cells with Ad-p16

Following the growth of MCF-7 and ZR75.1 cells to approximately 50% confluence in 100 mm plates, they were infected with Ad-p16 at multiplicity's of infection (moi's) of 10, 50 and 100, and incubated at 37°C for 42 hours. Control cells were left uninfected. After incubation, the uninfected and Ad-p16-infected cells were harvested both for FACS analysis to determine cell cycle distribution and immunoblot analysis to determine the expression profiles of cell cycle regulatory proteins. Both uninfected MCF-7 and ZR75.1 cells showed the expected cell cycle distribution and regulatory protein expression profiles for logarithmic cellular populations and lacked p16 expression. Infection with Ad-p16 resulted in expression of very high levels of p16, together with nearly complete G1 arrest and cessation of growth. Representative data for MCF-7 cells are shown in Figure 1. The effect of p16 expression upon the cell cycle was also illustrated by the expression profiles of cell cycle regulatory proteins, including cyclin D1, which showed decreasing expression with increasing levels of p16. This implies that the binding of p16 to CDK4/6 proteins resulted in the rapid degradation of monomeric cyclin D1 in those cells infected with Ad-p16 at high moi's. In addition, cyclin A and B proteins, which are expressed on entry into S- and M-phase, respectively, were completely lacking in Ad-p16-infected cells (Figure 1). These experiments demonstrated that both MCF-7 and ZR75.1 cells were sensitive to p16 function, and therefore suitable cell lines for the analysis of the effects of regulated p16 expression upon the transformed properties of breast cancer cell lines.

Construction of p16 stable transfectants using the Tet-Off System

As described in the previous progress report, we chose to employ the Tet-Off System (Gossen et al., 1995) to introduce regulated p16 expression into the breast cancer cell lines since we predicted and have subsequently shown using the Ad-p16 construct, that high, constitutive levels of p16 expression result in sustained G1 phase arrest. The construction of stable "Tet" clones involves the sequential two-step transfection of (a) the "regulator" plasmid (pUDH15-1), which encodes the tet-controlled transcriptional activator (tTA); followed by (b) the "response" plasmid (pTET-SPLICE-p16), which encodes the p16 gene under the control of the Tet Operator (tet O), into MCF-7 and ZR75.1 cells. The tTA binds the tet O sequence and activates transcription of p16 in the absence of tetracycline or its analogue, doxycycline. We chose to use doxycycline in our experiments because of its longer halflife in culture compared with that of tetracycline.

Stable transfection of MCF-7 and ZR75.1 cells with the "regulator" plasmid, pUDH15-1

We initiated the transfection of MCF-7 and ZR75.1 cells with pUDH15-1 just prior to the submission of the previous progress report. To briefly reiterate, two 6-well (35 mm) plates containing MCF-7 and ZR75.1 cells were grown to 50-60% confluence at which time ten of the twelve wells were transfected with 2.4 μg/well of the pUDH15-1 plasmid encoding the tTA. Two wells were left untransfected. After 5 hours at 37°C, the lipid/DNA mixture was removed from the cells and replaced with medium containing 10% serum. The cells were grown for a further 48 hours at 37°C before each well (including the two wells containing untransfected cells) was harvested and its cells transferred to one 100 mm plate containing medium supplemented with 10% serum and 500 μg/ml G418. The medium was changed three times per week and after two to three weeks, each plate containing transfected cells harbored approximately 15-20 discrete colonies. As predicted the untransfected cells lacking G418 resistance were killed. Continuing with these procedures in the current grant period, a total of 56 G418-resistant colonies from the MCF-7 transfection and 40 colonies from the ZR75.1 transfection have been isolated by "ring cloning". Cells were transferred to 25 cm^2 flasks and at approximately 50% confluence, the cells were harvested for DNA isolation and preparation of frozen stocks, leaving the remainder to grow in culture. The DNA was subjected to PCR analysis using primers that we designed to the tTA sequence (see Materials and Methods) in order to identify those that contained the pUDH15-1 plasmid. Of the 56 MCF-7 clones assessed by PCR, 20 contained the integrated plasmid, and of the 40 ZR75.1 clones assessed, 6 contained the plasmid.

Functional analysis of pUDH15-1 transfectants

To ascertain if the pUDH15-1 plasmid was functional in those clones that were shown to contain it by PCR, we transiently transfected pTET-SPLICE-p16 into 6 representative MCF-7 and ZR75.1 clones (that exhibited different growth rates) by lipid-

mediated transfection. Each of the clones were grown to approximately 50-60% confluence in each of four 35 mm wells, at which time two of the four wells were transfected with 2.4 μg/well pTET-SPLICE-p16, and two were left as untransfected controls. After 5 hours at 37°C the lipid/DNA mixture was aspirated off the cells. To two of the wells containing transfected and untransfected cells, respectively, we added medium containing 10% serum and 500 μg/ml G418 (to maintain the presence of the pUDH15-1 plasmid) plus 1 μg/ml doxycycline (to suppress tTA activation of p16 expression), and to the remaining two pairs, the identical medium but lacking doxycycline (to allow tTA activation of p16 expression) was added. Following 24 hours at 37°C, the cells were harvested for p16 expression by immunoblot analysis. Not surprisingly, we observed clonal variation with regard to the degree of p16 induction ranging from none at all, to very strong induction resulting in levels of p16 expression equivalent to or greater than that shown by the p16 overexpressing breast cell line, HBL-100 (Todd et al., manuscript in preparation). In the presence of doxycycline, however, there was no expression of p16, indicating the tightly-regulated, doxycycline-dependence of the system. Of the 6 MCF-7 clones assessed, 4 showed p16 induction, and of the 6 ZR75.1 clones assessed, 3 showed p16 induction. Representative data from MCF-7 and ZR75.1 clones expressing high, moderate and undetectable levels of p16 are shown in Figure 2. Variation in the level of p16 expression likely resulted from differences in the copy number and site/orientation of integration of the pUDH15-1 plasmid in the various independent clones analyzed. Two possible explanations for the lack of p16 expression in some of the clones are (a) cis effects at the site of plasmid integration that suppressed expression of the tTA; or (b) the expression of a non-functional tTA that was unable to bind to the tet O sequence. Based upon the results of the transient transfections, we chose one MCF-7 and one ZR75.1 transfectant to stably transfect with the pTET-SPLICE-p16 plasmid. Clones MCF-7/15-1#11 and ZR75.1/15-1#3 which exhibited a moderate degree of p16 induction relative to HBL-100, were chosen because our objective was to express a level of p16 that would be sufficiently high to study its effect upon the transformed and tumorigenic properties of breast cancer cell lines, but not so great as to cause cells to arrest in G1 (as we observed following infection of cells with the Ad-p16 adenoviral construct).

Stable transfection of MCF-7/15-1#11 and ZR75.1/15-1#3 with pTET-SPLICE-p16

Because the pTET-SPLICE-p16 construct did not harbor antibiotic resistance, we chose to cotransfect it with the plasmid, pZeo (Invitrogen) which enable the selection of clones based upon resistance to zeocin. Prior to the transfection, we performed zeocin "kill-curve" assays on the two clones with concentrations ranging from 200 to 1000 μg/ml. The minimum concentration required to kill all untransfected cells within 8-10 days was determined to be 500 μg/ml. The MCF-7/15-1#11 and ZR75.1/15-1#3 cells were grown in 8 35 mm plates until 50-60% confluence. At this time, 6 of the 8 wells were cotransfected with 2.4 μg/well pTET-SPLICE-p16 and 0.12 μg/well pZeo, and the remaining 2 wells were left untransfected. After 5 hours at 37°C, the lipid/DNA mixture was removed from the cells. To 5 of the 6 wells containing transfected cells, medium containing 10% serum, 500 μg/ml G418

and 1 μg/ml doxycycline (to suppress p16 expression) was added, and to the remaining 3 wells, medium containing 10% serum, 500 μg/ml G418 but lacking doxycycline (to allow p16 expression) was added. The cells were grown for another 48 hours at 37°C before each well was harvested and the cells transferred to each of 8 100 mm plates. The 5 plates containing transfected cells that had been grown in the presence of doxycycline, were cultured in medium supplemented with serum, 500 μg/ml G418, 500 μg/ml zeocin and 1 μg/ml doxycycline, whereas the untransfected cells and the transfected cells previously grown in the absence of doxycycline were cultured in the identical medium to that described above but lacking in doxycycline.

Isolation of MCF-7/15-1#11 p16 transfectants

At 7 days post-transfection, all 5 of the 100 mm dishes containing cells cultured in the presence of doxycycline were approximately 40-50% confluent and were therefore replated at lower densities to enable the isolation of discrete independent colonies. By contrast, those transfectants cultured in the absence of doxycycline failed to proliferate and remained non-dividing for the duration of the experiment. We attribute the arrest and eventual senescence of these cells to the lack of doxycycline-mediated suppression of p16 expression. As expected, the two plates containing the untransfected cells were killed in the presence of zeocin.

At 3-5 weeks post-transfection, a total of 94 colonies were isolated by "ring cloning" and transferred to 25 cm^2 flasks. At approximately 50-60% confluence, the cells were harvested for DNA isolation and preparation of frozen stocks, leaving the remainder to grow in culture. Because the p16 gene is homozygously deleted in the MCF-7 cell line, we were able to identify those clones that contained the pTET-SPLICE-p16 plasmid by subjecting the DNA samples to PCR using primers designed to amplify exon 2 of the p16 gene (Hussussian et al., 1994). Of the 94 clones screened by PCR, 29 showed amplification of p16.

Isolation of ZR75.1/15-1#3 p16 transfectants

Unlike the MCF-7/15-1#11 transfection experiment, at 7 days post-transfection, the ZR75.1/15-1#3 transfectants cultured in the presence and absence of doxycycline appeared unhealthy with a significantly suppressed growth rate. In an attempt to improve the yield of transfectants, we attempted to optimize transfection efficiency by varying the amounts and ratios of pTET-SPLICE-p16 and pZeo DNA used, and altering the concentration of zeocin used for the selection of clones. Manipulation of latter variable proved to be critical in the successful isolation of healthy ZR75.1/15-1#3 transfectants. Briefly, the transfection procedure was repeated exactly as described above with the exception that the cells were transferred into 100 mm plates containing medium supplemented with 200 μg/ml zeocin instead of 500 μg/ml zeocin at 48 hours post-transfection. After 5 days in the less stringent antibiotic selection conditions, the concentration of zeocin was increased to 500 μg/ml used in the MCF-7/15-1#11 experiments. This proved a more successful strategy, and resulted in the

isolation of 27 zeocin-resistant colonies. DNA was not prepared from these clones since the parent cell line, ZR75.1 contains at least one copy of the p16 gene, in its methylated non-transcribed state (Todd et al., manuscript in preparation), that would be detected in all of the clones by the exon 2-specific oligonucleotides. We conclude that the initial selection of the ZR75.1/15-1#3 p16 transfectants in the lower zeocin concentration proved successful as it enabled cells to grow under less stringent conditions until the expression of zeocin resistance by the integrated pZeo plasmid was complete.

Analysis of MCF-7/15-1#11 and ZR75.1/15-1#3 transfectants for p16 expression

All 29 of the zeocin-resistant MCF-7/15-1#11 clones that demonstrated p16 amplification, and 22 of the 27 zeocin-resistant ZR75.1/15-1#3 clones, were assessed for doxycycline-dependent expression of p16 protein. Each of the clones were grown in two 35 mm wells either in the presence or absence of doxycycline for 5 days. The cells were then harvested for protein isolation and immunoblot analysis of p16 expression. As expected, based upon the results of the p16 transient transfections, discussed above, the induction of p16 expression in the absence of doxycycline varied widely among the clones. Indeed, of the 29 MCF-7/15-1#11 clones assessed, 4 exhibited high levels of p16 expression, 3 exhibited moderate levels, 5 exhibited low levels and 17 showed no induction of p16 expression at all. Similarly of the 22 ZR75.1/15-1#3 clones assessed, 3 exhibited high levels of p16 expression, 4 exhibited moderate levels, 1 exhibited low levels and 14 showed no p16 induction (see examples in Figure 3). Furthermore, all of the clones that demonstrated moderate to high expression of p16 also underwent a shift from the hyperphosphorylated (inactive) form of pRb which predominated in the presence of doxycycline to a predominantly hypophosphorylated (active) form of pRb in the absence of doxycycline (data not shown). However, in contrast to the complete cessation of growth in cells infected with Ad-p16 adenovirus and expressing extremely high levels of p16, these cells were capable of continued growth. These data indicate that a threshold level of exogenous p16 expression is capable of reducing cyclin D1/CDK4/6 phosphorylation of pRb, and thus contributing to regulation at the G1/S boundary, rather than causing a total inhibition of cell cycle progression as seen in Ad-p16 infected cells. The majority of clones displayed doxycycline-dependence with regard to the degree of p16 expression induced. However, those clones that expressed moderate to high levels of p16 in the absence of doxycycline, also expressed barely detectable to low levels of the p16 protein in the presence of doxycycline. This finding indicates a slight "leakiness" in the Tet-Off system.

Owing to the large number of clones isolated, we have been able to identify three transfectants from each of the two cell lines, that express low, moderate and high levels of p16 expression in the absence of doxycycline with zero to barely detectable expression in the presence of doxycycline. We have employed these clones to analyze the effect of different levels of p16 expression upon the *in vitro* and *in vivo* transformed properties of the breast cancer cell lines, MCF-7 and ZR75.1. To date, we have focused upon the analysis of the following MCF-7 clones: MCF-7/15-1/p16#29, which exhibits low p16 expression; MCF-

7/15-1/p16#69, which exhibits moderate p16 expression; and MCF-7/15-1/p16#13, which exhibits high p16 expression (Figure 3). The latter two clones express low levels of "leaky" p16 expression in the presence of doxycycline. Owing to the technical problems associated with transfection of ZR75.1/15-1#3 with pTET-SPLICE-p16, we have only just initiated the analysis of the clones, ZR75.1/15-1/p16#12, which exhibits low p16 expression; ZR75.1/15-1/p16#7, which exhibits moderate p16 expression; and ZR75.1/15-1/p16#10, which exhibits high p16 expression. These data will be presented in the final progress report.

Effect of regulated p16 expression upon cell cycle distribution of breast cancer cells

Our analysis of Ad-p16-infected MCF-7 and ZR75.1 cells showed that extremely high levels of p16 protein were capable of causing essentially complete G1 arrest. Our goal, therefore, was to determine the cell cycle distribution in the clones, MCF-7/15-1/p16#29, MCF-7/15-1/p16#69, and MCF-7/15-1/p16#13, following induction of the low, moderate and high (but not extreme) levels of p16 expression seen in these cells. The parent cell line, MCF-7, and each of the three clones were grown in the presence and absence of medium containing 1 μg/ml doxycycline for 7 days, then harvested both for FACS and p16 and pRb expression analysis. Immunoblot analysis indicated the expected degree of p16 induction in the clones grown in the absence of doxycycline (Figure 4). In addition, the moderate and high induction of p16 expression shown by clones MCF-7/15-1/p16#69 and MCF-7/15-1/p16#13, correlated with the presence of the active (hypophosphorylated) species of the pRb protein (data not shown). When these data were compared directly with the cell cycle distribution profiles, it became clear that at least moderate expression of p16 was sufficient to cause a significant accumulation of cells in G1 phase (Figure 4). By contrast, the cell cycle distribution profiles for clone, MCF-7/15-1/p16#29, grown in the presence and absence of doxycycline, were virtually identical. These data confirm that the p16 protein expressed by the MCF-7 transfectants is functional and capable, at a threshold level of inhibiting phosphorylation of pRb protein by cyclin D1/CDK4/CDK6, thereby regulating entry into S-phase. To provide further biochemical evidence for this conclusion, we plan to examine the coimmunoprecipitation of p16 with CDK4/6 in extracts isolated from each of the three clones grown in the presence and absence of doxycycline for 7 days.

Effect of regulated p16 expression upon anchorage-independence of breast cancer cell lines

One of the characteristics exclusive to tumor cells is their ability to grow in the absence of anchorage to substrate. It is not known whether p16 plays a role in anchorage dependence as the underlying mechanisms have yet to be fully elucidated. To assay the effect of p16 expression upon the ability of MCF-7 cells to grow in an anchorage-dependent manner, we subjected the clones, MCF-7/15-1/p16#29, MCF-7/15-1/p16#69, and MCF-7/15-1/p16#13, to soft agar analysis. Both the parent cell line, MCF-7, and each of the three transfectants were grown in two 75 cm^2 flasks in the presence and absence of 1 μg/ml

doxycycline-containing medium for 7 days, to allow p16 expression to be switched "on" to its full extent in the cultures lacking doxycycline. The cells were then harvested both for p16 expression analysis and soft agar assays. For the analysis of p16 expression, protein extracts were prepared from the same cells as those used for soft agar analysis. All three of the clones showed the expected degree of p16 induction in the absence of doxycycline. For the soft agar assays, MCF-7 cells were suspended in an agar/medium/10% serum mixture plus or minus 1 μg/ml doxycycline, and the transfectants were suspended in an identical mixture supplemented with 500 μg/ml G418 and 500 μg/ml zeocin plus or minus 1μg/ml doxycycline. A total of 1.25×10^4 cells of each cell line were plated onto a layer of agar/medium/10% serum in 8 replicate 60 mm plates. Fresh medium containing the appropriate antibiotic selection plus or minus doxycycline was added to the plates twice per week. After 2.5-3 weeks, the plates were scored for the growth of colonies in soft agar.

The MCF-7 parent cells grew successfully both in the presence and absence of doxycycline forming large, healthy colonies with no evidence of growth suppression in the absence of anchorage. MCF-7/15-1/p16#29 cells also grew to form healthy colonies, though they were fewer and smaller in size than those formed by the MCF-7 parent. Plates containing MCF-7/15-1/p16#29 cells grown in the absence of doxycycline looked similar to those grown in the presence of doxycycline but upon closer scrutiny appeared to contain fewer healthy colonies and more single, inviable cells. The most marked contrast to the MCF-7 parent plates was exhibited by clones MCF-7/15-1/p16#69, and MCF-7/15-1/p16#13. Growth in both the presence and absence of doxycycline resulted in a high frequency of cell death as manifested by the preponderance of inviable single cells and small clusters of 2-8 cells on all of the plates. The observation of an insignificant difference between the suppression of growth in soft agar exhibited by cells grown either in the presence or absence of doxycycline suggests that the level of "leaky" p16 expression exhibited by these two clones in the presence of doxycycline was sufficient to inhibit the growth of MCF-7 cells in soft agar. In keeping with this possibility, preliminary observations indicate that the Rb protein is predominantly in the hypophosphorylated, (active) state in these cells when grown in soft agar, even in the presence of doxycycline. Indeed, even MCF-7/15-1/p16#29 cells grown in the presence of doxycycline which do not show detectable expression of p16 by immunoblot appear to be somewhat growth compromised. Two possible explanations are (a) that a very low level of p16 protein may be expressed in clone MCF-7/15-1/p16#29 in the presence of doxycycline, giving rise to an incomplete restoration of anchorage-dependent properties; or (b) that the transfectants themselves, are modified in such a way as to suppress their growth in soft agar. To test these two hypotheses, we are in the process of analyzing MCF-7 transfectants containing both pUDH15-1 and pTET-SPLICE-p16 plasmids that fail to express p16 protein for their ability to grow in soft agar. Owing to the lack of p16 protein expression in these clones, we predict that they should grow in soft agar. Failure to grow may indicate the contribution of other factors to anchorage-dependent growth.

Effect of regulated p16 expression upon tumorigenicity of breast cancer cells

We are currently investigating the effect of the different levels of p16 expression exhibited by the clones MCF-7/15-1/p16#29, MCF-7/15-1/p16#69, and MCF-7/15-1/p16#13, upon tumorigenicity in nude mice, and will shortly begin the analysis of the clones ZR75.1/15-1/16#12, ZR75.1/15-1/16#7, and ZR75.1/15-1/16#10. The growth of MCF-7 tumors in nude mice is estrogen-dependent. Thus, to induce tumor development following the sub-cutaneous injection of MCF-7 or MCF-7 transfectant cells into nude mice, requires the prior implantation of a 1.7 mg pellet of 17 ß-Estradiol (Innovative Research of America) that releases hormone over a 90-day period to give constant levels in the blood of 500-600 pg/ml. We are in the process of growing the MCF-7 parent cell line in addition to the three MCF-7 transfectants in the presence and absence of 1 µg/ml doxycycline prior to the injection into nude mice (harboring estrogen pellets). A critical experiment that most closely reflects the clinical situation of a patient with an established, vascularised tumor undergoing gene therapy will then be performed:

Each of the three transfectants that have been growing in the presence of doxycycline will be injected (at 5 x 10^6 cells per mouse) into 4 mice that harbor a subcutaneous, slow release doxycycline pellet (Innovative Research of America), to maintain suppression of p16 expression. The tumors will be allowed to grow until they reach 1 cm x 1cm, at which time, the doxycycline pellet will be removed from two of the four mice thus allowing p16 expression to be turned "on" in these tumor cells. The doxycycline pellet will not be removed from the remaining two mice thus maintaining suppression of p16 expression in the tumors. The tumors will be allowed to grow for 3 months at which time the mice will be euthanized and the tumors respected, weighed and analyzed for the expression of p16, pRb and other G1 phase regulatory proteins. In this way, we will be able to determine the growth regulatory effects of turning "on" p16 expression to low, moderate and high levels in the breast tumors.

The controls for this experiment will include (a) the injection of duplicate sets of mice, lacking the doxycycline pellet, with each of the three transfectants clones MCF-7/15-1/p16#29, MCF-7/15-1/p16#69, and MCF-7/15-1/p16#13 (at 5 x 10^6 cells per mouse) that have been cultured in the absence of doxycycline for 7 days prior to injection. In these cells, the p16 gene will have been turned "on" to low, moderate and high levels, respectively, and should remain so in the mice. We will then be able to determine if the preexisting expressing of p16 is sufficient to suppress the growth of tumors at all following injection and compare these data with those obtained in the above experiments; and (b) the injection of four nude mice with the untransfected parent cell line, MCF-7 (at 5 x 10^6 cells per mouse), two of which will contain the doxycycline pellet. This last control will indicate if the presence of the pellet effects the growth of tumors which we predict will not be the case.

We hope that these experiments will identify which (if any) of the three MCF-7 transfectants are suppressed for tumor formation following the activation of p16 expression

in nude mice. In this way, we will be able to ascertain (a) if p16 expression is capable of suppressing the *in vivo* tumorigenicity of breast cancer cell lines; and is so, (b) the minimal level of p16 protein expression necessary to elicit such suppression.

Other Studies in Progress

The studies reported above on the contribution of p16 expression to tumorigenicity in breast cancer cells have been the major focus of our efforts during this period of the grant. In addition, several other studies on aspects of cell cycle regulatory defects in breast cancer have been in progress during this period and will be continued through the remainder of the grant. The current status of these studies is briefly indicated below, and a full description of this work will be included in the final progress report.

Mechanisms of p16 inactivation in breast tumor tissues

The previous progress report presented the analysis of a panel of 10 pairs of normal and tumor breast tissues for the expression of the G1 phase regulatory proteins, cyclin D1, pRb and p16 proteins. Like the breast cancer cell lines previously analyzed, all 10 tumors demonstrated a correlation between cyclin D1 and pRb expression. Specifically, 8 of the 10 tumor tissue extracts expressed pRb, overexpressed cyclin D1, and failed to express detectable levels of p16 protein, relative to the corresponding normal tissue controls. The lack of p16 protein expression in the tumor tissues suggested the possible inactivation of the p16 gene. We therefore subjected DNA isolated from microdissected cells corresponding to the 10 pairs of normal and tumor cells, to methylation-specific PCR (MSP) analysis at the p16 locus. Normal and tumor DNA was modified overnight using sodium bisulfite to convert all of the unmethylated cytosines to uracil. The resulting DNA was then subjected to PCR analysis using oligonucleotides designed from the promoter of the p16 gene that were specific for wild type (unmodified), methylated and unmethylated DNA (Herman et al., 1996). As expected, all of the normal samples demonstrated amplification using the unmethylated-specific primers only. Surprisingly, however, all 10 of the tumor samples also underwent amplification with the unmethylated primers. This result indicated that none of the tumors were homozygously deleted for the p16 gene since all were successfully amplified, and that the p16 gene was not transcriptionally silenced in these tumors due to promoter methylation. The lack of p16 gene inactivation by deletion or methylation leaves open the possibilities that (a) the p16 gene is inactivated by another mechanism including deletion of one allele and mutational inactivation of the remaining allele; or (b) the p16 is expressed in the tumor tissues at a very low level that is not detectable by immunoblot analysis. To address the first possibility, we are in the process of analyzing the microdissected normal and tumor samples for loss of heterozygosity at five loci closely linked to the p16 gene. Those tumors which show allelic loss at one or more loci will be likely also to have lost one copy of the p16 gene, and will therefore be analyzed for mutations within the remaining copy of the p16 gene. To address the second possibility, we plan to obtain RNA from the 10 pairs of microdissected

normal and tumor tissues, and subject it to RT-PCR analysis to look for evidence of low level p16 expression.

Construction of stable antisense cyclin D1 transfectants using the Tet-Off system

The previous progress report discussed our attempts to suppress the expression of cyclin D1 in the breast cancer cell lines, MCF-7 and ZR75.1 transiently transfected with both pUDH15-1 and pTET-SPLICE-antisense cyclin D1 plasmids. Using a variety of plasmid DNA and lipid concentrations, we were unable to acheive detectable suppression of cyclin D1 protein expression. Since then, we have isolated stable MCF-7 and ZR75.1 transfectants containing the pUDH15-1 plasmid, and have attempted to transiently transfect these clones with pTET-SPLICE-antisense cyclin D1 construct. Again, using varying amounts of plasmid and lipid, we have been unable to demonstrate significant suppression of cyclin D1 protein expression. One possible explanation for this result is that only a subpopulation of transiently transfected cells contained the pTET-SPLICE-antisense cyclin D1, such that a reduction in cyclin D1 expression was not detectable by immunoblot analysis.

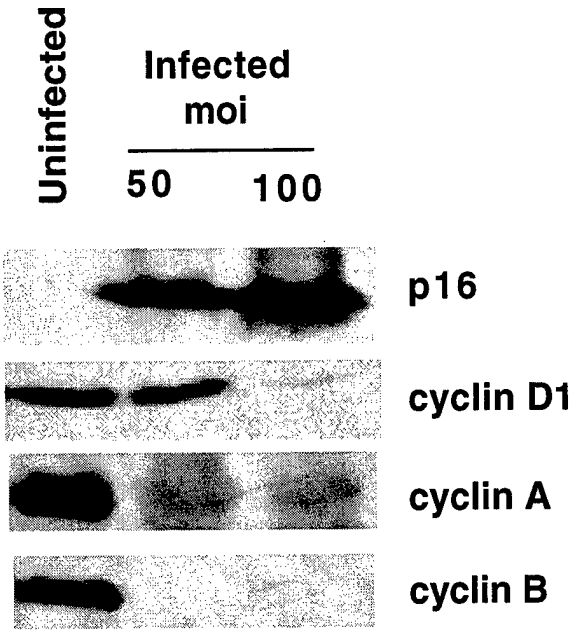
We are currently in the process of isolating MCF-7 clones that are stably transfected with both the pUDH15-1 and pTET-SPLICE-p16 plasmids. The "response" plasmid, pTET-SPLICE-antisense cyclin D1, was cotransfected with pZeo into MCF-7/15-1#11 (that contains stably integrated pUDH15-1), and selected for resistance to both G418 and zeocin in 100 mm plates. We are currently isolating individual colonies by "ring cloning" and expanding in culture, prior to analysis of cyclin D1 expression and tumorigenic properties in the presence and absence of doxycycline.

Conclusions

In previous years we have characterized a number of defects in the expression of cell cycle regulatory components in breast cancer cells, and established some of the mechanisms responsible for causing these defects. In the current year we have turned our major focus towards demonstrating the contribution of these defects to the tumorigenic properties of breast cancer cells, in order to provide potential targets for therapy. In particular, we have focused on the effects of restoring the expression of the tumor suppressor p16 in breast cancer cells, using an expression system regulated by the presence of the tetracycline analog doxycycline. After establishing the feasibility of this system in breast cancer cells using transient transfection, we have in the current year generated a large number of stable transfectants in which p16 expression is regulated by doxycycline. From these stable transfectants we have isolated three MCF-7 and three ZR75.1 breast cancer cell clones, which express p16 at low, moderate and high levels. These cells will be used for study of changes in tumorigenic properties due to the expression of p16, namely growth in soft agar and tumor formation in nude mice. Currently we have demonstrated that MCF-7 cells stably transfected with constructs expressing p16 are impaired or have lost the ability to grow in soft agar, indicating that defects in p16 expression may make a critical contribution to the tumorigenicity of these cells. Since loss of growth in soft agar of these cells was found not to be dependent on induction of expression of p16, very low levels of p16 may be sufficient to suppress tumorigenicity in these cells. However, additional controls will be required to establich unequivocally that loss of growth in soft agar in these cells is in fact due to p16 expression.

References

Gossen et al. (1995). Science 268:1766-1769.
Herman et al. (1996). Proc. Natl. Acad. Sci. USA 93:9821-9826
Hussussian et al. (1994). Nature Genetics 8:15-21.
Krishan. (1975). J. Cell Biol. 66:188.
Laemmli, U. K. (1970). Nature (London) 227:680-685.
Schrump et al. (1996). Cancer Gene Therapy 3 (6):357-364.



% G1	61	94	95
% S	29	6	3
% G2/M	10	0	2

Figure 1 Infection of breast cancer cells with an adenoviral-p16 construct, Ad-p16, results in a high level of p16 expression and G1-phase arrest. Logarithmic phase MCF-7 cells were infected with Ad-p16 at moi's of 50 and 100, or left uninfected. After 42 hours at 37°C, cells were harvested for FACS and immunoblot analyses. Uninfected cells showed the expected cell cycle distribution and expression of cyclin proteins for a logarithmic population, in addition to the lack of p16 expression and overexpression of cyclin D1 protein. Infected cells, by contrast, expressed extremely high levels of p16 protein which resulted in the degradation of cyclin D1 protein and G1 accumulation. The latter is illustrated by the significant loss of expression of the S and G2/M phase-specific cyclins, A and B, respectively.

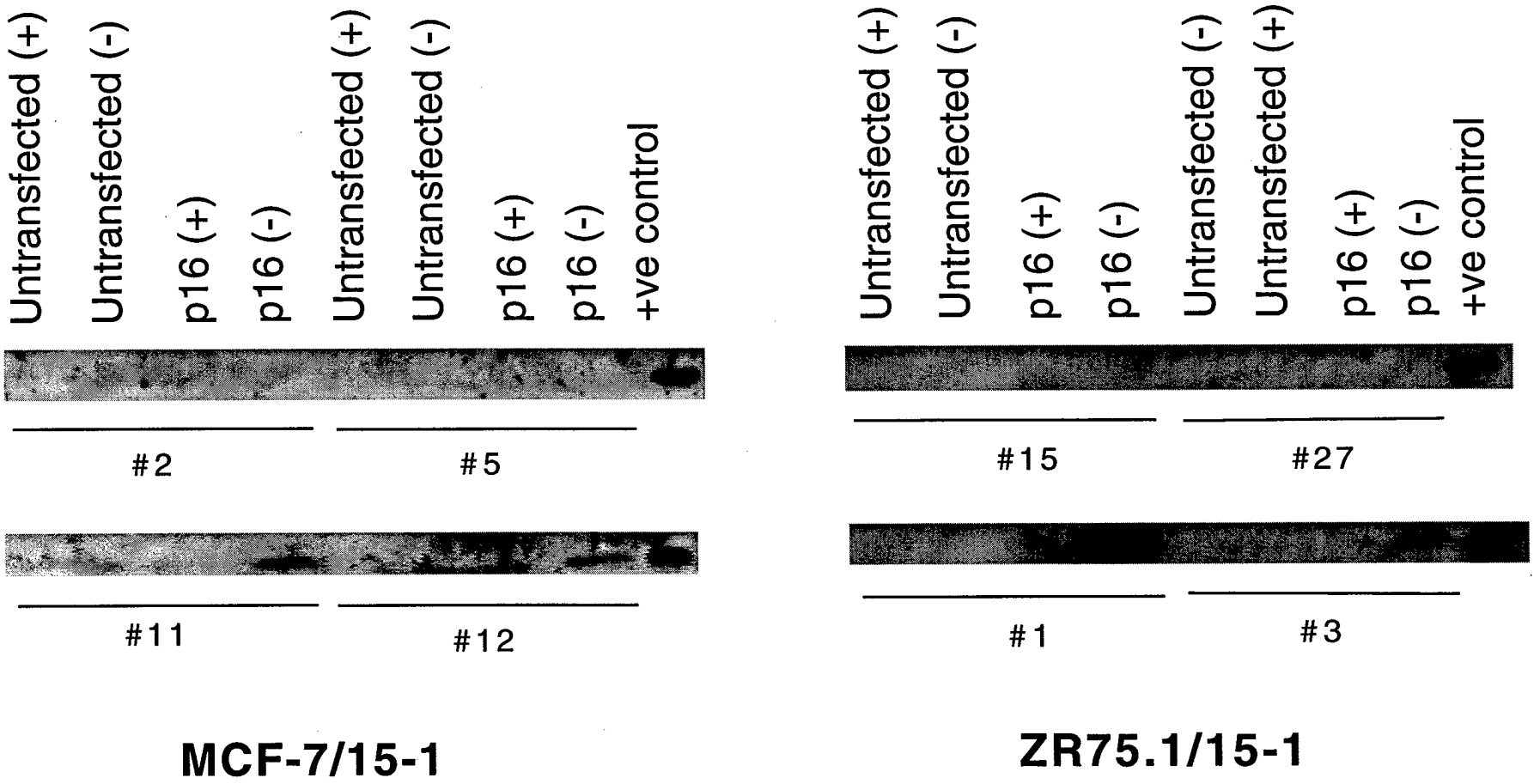


Figure 2 Induction of p16 expression by tTA

MCF-7 and ZR75.1 clones stably transfected with pUDH15-1 were subjected to transient transfection with pTET-SPLICE-p16, and grown in the presence (+) or absence (-) of doxycycline for 24 hours. The resulting transfectants showed a wide range of p16 expression levels in the absence of doxycycline, a representative selection of which are shown in the figure.

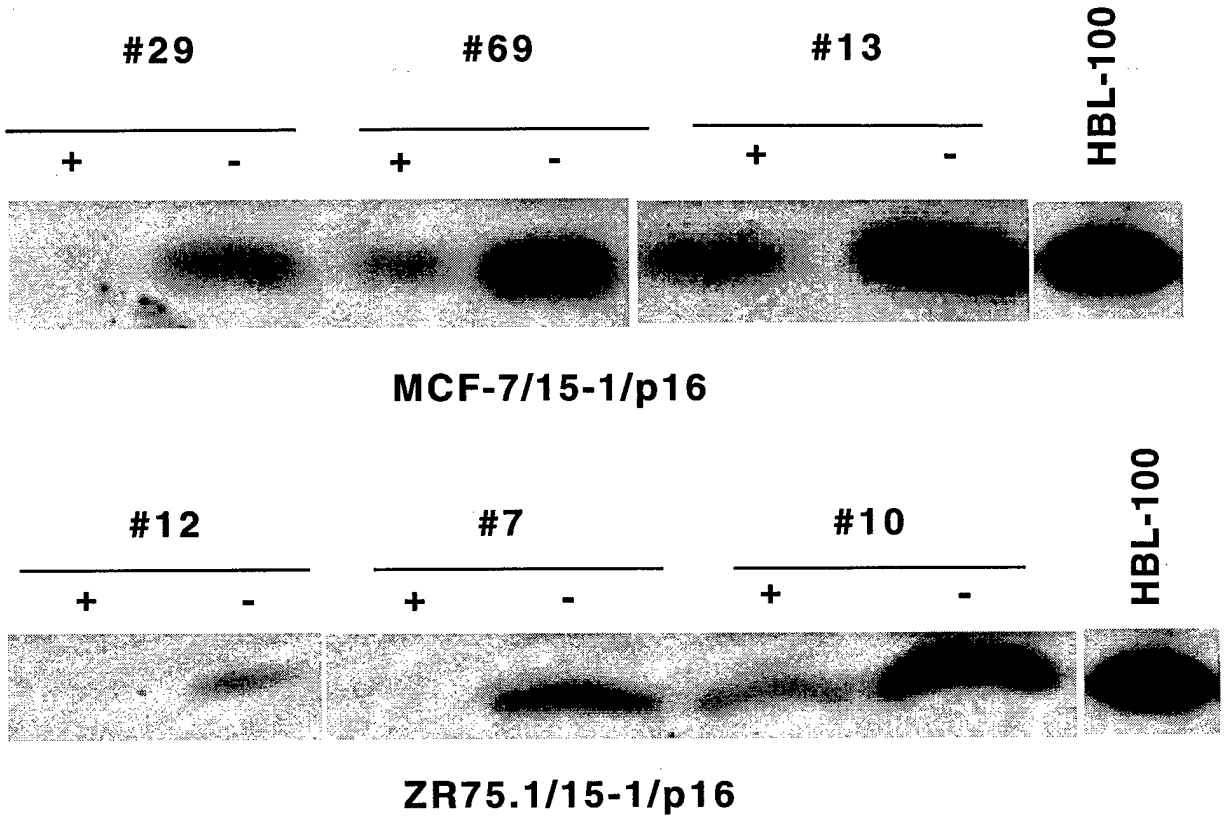


Figure 3 Induction of p16 expression in MCF-7 and ZR75.1 clones stably transfected with pUDH15-1 and pTET-SPLICE-p16.

MCF-7 and ZR75.1 "double stable" clones were grown in the presence and absence of doxycycline for 7 days then subjected to immunoblot analysis using an antibody to p16. The clones exhibited a range of p16 expression following the removal of doxycycline (and in some cases, a low level of expression in the presence of doxycycline). Representative clones expressing low, moderate and high levels of p16 induction (relative to HBL-100 which exhibits overexpression of p16), are shown in the figure.

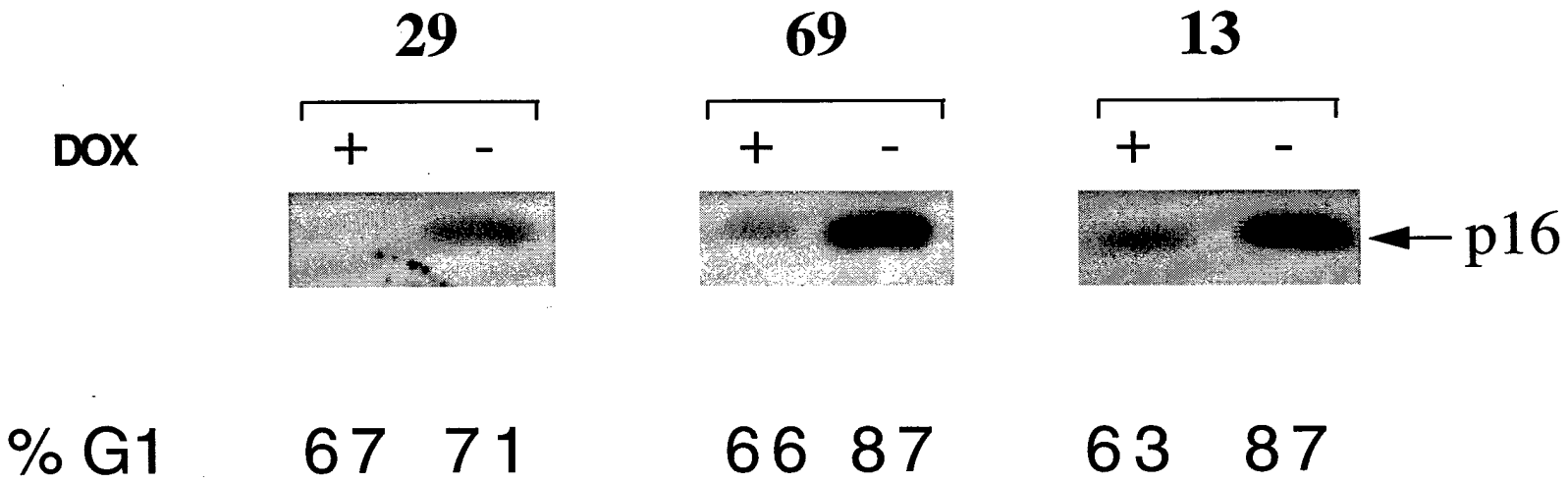


Figure 4 G1 accumulation following doxycycline-induced p16 expression in MCF-7 breast cancer cell line transfectants

Three MCF-7 "Tet" clones, MCF-7/15-1/p16#29, MCF-7/15-1/p16#69, and MCF-7/15-1/p16#13, were grown in the presence or absence of doxycycline for 7 days prior to immunoblot and cell cycle distribution analyses. The induction of moderate and high expression of p16 in the absence of doxycycline (in clones #69 and #13, respectively), correlated with a significant accumulation of cells in G1 phase.